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**The Effect of Acute Oral Phosphatidic Acid Ingestion on Myofibrillar Protein Synthesis
and Intracellular Signaling in Older Males.**

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ABSTRACT

Background: Age-related muscle loss (sarcopenia) may be driven by a diminished myofibrillar protein synthesis (MyoPS) response to anabolic stimuli (i.e. exercise and nutrition). Oral phosphatidic acid (PA) ingestion has been reported to stimulate resting muscle protein synthesis in rodents, and enhance resistance training-induced muscle remodelling in young humans. **Purpose:** This study examined the effects of acute oral PA ingestion on resting and exercise-induced MyoPS rates in older individuals. **Methods:** Sixteen older males performed a bout of unilateral leg resistance exercise followed by oral ingestion of 750mg of soy-derived PA or a rice-flour placebo (PL) over 60 min post-exercise. A primed-continuous infusion of L-[ring-¹³C₆]-phenylalanine with serial muscle biopsies was used to determine MyoPS at rest and between 0-150 and 150-300 min post-exercise. **Results:** Plasma [PA] concentrations were elevated above basal values from 180-300 min post-exercise in PA only ($P = 0.02$). Exercise increased MyoPS rates above basal values between 150-300 min post-exercise in PL ($P = 0.001$), but not PA ($P = 0.83$). Phosphorylation of p70S6K, rpS6, 4E-BP1 and Akt was elevated above basal levels in the exercised leg over 150-300 min post-exercise for PL only ($P = 0.018, 0.007, 0.011$ and 0.002 , respectively), and were significantly greater than PA ($P < 0.01$ for all proteins). The effects of oral PA ingestion on proteolytic signaling markers was equivocal. **Conclusions:** Acute oral phosphatidic acid ingestion appears to interfere with resistance exercise-induced intramuscular anabolic signaling and MyoPS in older males and, therefore, may not be a viable treatment to counteract sarcopenia. Clinicaltrials.gov registration no: NCT03446924

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INTRODUCTION

Age-related loss of skeletal muscle mass and strength, also termed sarcopenia [1], is associated with premature mortality [2] and leads to myriad adverse health consequences such as impaired functional capacity [3], increased risk of fractures [4], and metabolic disease [5]. Whilst the incidence of age-related muscle wasting has been observed in people as young as 45 y, its prevalence increases with advancing age [6]. The healthcare costs associated with sarcopenia are extensive and, in the context of a rapidly expanding global ageing population, are expected to increase considerably in the coming decades [7].

Skeletal muscle proteostasis is dependent on the equilibrium between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). One of the most effective strategies to attenuate the progression of sarcopenia is resistance exercise (RE) training [8-10]. In young individuals, RE robustly increases MPS by 2 to 3-fold [11], thereby enhancing overall net protein balance for muscle hypertrophy [12, 13]. However, the acute MPS response to RE is blunted in older compared with younger individuals [14, 15], which may explain the impaired muscle remodelling response to prolonged training in older age [15, 16]. The age-related blunting of the muscle anabolic response to RE may be underpinned by impairments in ribosomal biogenesis and/or translational efficiency in the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway [14, 15, 17]. Collectively, these data have galvanized the search for strategies to abolish the impaired muscle anabolic response to RE and maximize the therapeutic benefits of this non-pharmacological intervention in the old.

The lipid second messenger phosphatidic acid (PA) has been touted as an important factor in the regulation and activation of mTORC1 for MPS. Specifically, mechanical contraction increases the activity of diacylglycerol kinase ζ to convert diacylglycerol into PA, which in turn, binds mTORC1 directly at the FKB12 rapamycin binding (FRB) domain [18, 19], increasing its activity and ultimately enhancing protein translational efficiency [20].

Exogenous PA administration in cell culture is converted to lysophosphatidic acid [21], thereby inactivating the tuberous sclerosis complex (TSC1/2) through the extracellular regulated kinase (ERK) pathway to bolster mTORC1-mediated signaling [22]. Mechanistically, acute oral PA ingestion was reported to stimulate mTORC1-mediated signaling with a tendency to increase MPS in rodents [23]. Given that mechanical load-induced PA and exogenous PA appear to converge on mTORC1 through distinct proximal pathways, exogenous PA provision (via oral ingestion) may modulate the anabolic response to RE in older individuals [24]. Indeed, studies in healthy young humans report that orally ingested PA enhances strength and lean body mass gains when consumed daily during prolonged RE training [25, 26]. However, there is a need for further *in vivo* human investigation of the bioavailability and intramuscular mechanisms through which this purported ‘nutraceutical’ compound acts before introduction to the older population [27].

Therefore, the primary aim of the present study was to establish the effects of acute oral PA ingestion on rates of myofibrillar protein synthesis (MyoPS) and intramuscular signaling at rest and in response to a bout of RE in older individuals. We hypothesised that PA ingestion alone would not stimulate MyoPS, but would modulate RE-induced intramuscular signaling and MyoPS rates in older individuals compared with a placebo.

METHODS

Participants

Sixteen older males were recruited for the present study (age 68.9 ± 2.8 yrs, range 65-75 yrs). All participants were recreationally active and deemed healthy based on their responses to a general health questionnaire. Participants suffering from uncontrolled hypertension or generalised neuromuscular, metabolic or cardiovascular diseases were excluded from the study. Furthermore, taking part in regular structured resistance exercise whilst partaking in

the current study, smoking, and consuming non-steroidal anti-inflammatory drugs or any medication that might interfere with muscle metabolism, rendered the participant ineligible to participate. Prior to obtaining written consent, participants were informed of the purpose and methodology of the study. Ethical approval was obtained through the Solihull Research Ethics Committee (15/WM/0228). The study conformed to the latest guidelines set by the Declaration of Helsinki (7th edition). This trial is registered at Clinicaltrials.gov registration no: NCT03446924

Experimental design

Following an initial screening, participants visited the laboratories of the School of Sport, Exercise and Rehabilitation Sciences (SportExR) on two separate occasions. The study was parallel-designed and single-blinded, with participants randomized to receive either the placebo control (PL; n = 8) or phosphatidic acid treatment (PA, n = 8). During the initial visit, anthropometric characteristics and isotonic leg strength were determined. Approximately one week after this initial visit, participants underwent an experimental trial to determine the muscle anabolic properties of supplemental PA. The experimental trial consisted of a stable isotope amino acid infusion combined with serial muscle biopsies to determine the MyoPS response to PA or PL consumption alone, or in combination with resistance exercise.

Preliminary Assessments

Body mass and height: Participant body mass was recorded in loose clothing and without shoes to the nearest 0.1kg using a digital balance scale. Height was determined to the nearest 0.1cm using a stadiometer.

Body composition: Following determination of body mass and height, participants underwent a dual energy x-ray absorptiometry scan (DXA) (Discovery DXA Systems, Hologic Inc., Bedford, MA) to determine whole-body and regional bone mineral density, fat- and fat-free mass. DXA scans were performed after a ~10 h overnight fast. Participants rested supine on the scanner in loose clothing with their feet positioned at shoulder width apart and held in place with micropore tape. Each scan took 7 min and was analysed by a trained DXA operator.

Isotonic leg strength: Participant knee extensor one repetition maximum (1RM) strength was estimated in the dominant leg using a leg extension machine (Cybex VR-3, Medway, MA, USA) in order to determine the appropriate load of 75% 1RM for the subsequent experimental trial. Briefly, prior to exercise commencement, a baseline blood pressure measurement was obtained to ensure participants were normotensive (diastolic blood pressure 60-90 mmHg, systolic blood pressure 120-140 mmHg) to reduce the risk of adverse events during heavy-load, fatiguing RE. Following blood pressure assessment, participants initiated a self-selected 1-set warm-up after which the exercising load was gradually increased over subsequent sets until participants were unable to perform >10 repetitions. This final load was used to estimate knee extensor 1RM strength via the Brzycki equation [28]. The increment in loading was based on subjective ratings of exercise intensity, which were recorded immediately after each lifting attempt using a modified Borg category-ratio scale (CR-10) [29]. Each exercise set was separated by 2 min of passive rest. Strength assessments were led by a trained strength and conditioning specialist.

Dietary and activity control: Participants were asked to fast 10 hours overnight for both the initial and experimental trial visit, but were provided with a standardised meal on the evening prior to the experimental trial only. The meal contained ~787 kcal, comprised of ~19% protein (~37.5 g), ~46% carbohydrate (~90.7 g) and 35% fat (30.8 g). Participants were asked

to abstain from alcohol and caffeine for 24 h prior to the experimental trial. Furthermore, participants were asked to refrain from strenuous exercise for 48 h prior to the experimental trial.

Experimental Trial

Following a 10-hour overnight fast, participants returned to the SportExR laboratory at 0630 h following a ~10 h overnight fast. Upon arrival, a 21G cannula was inserted in an antecubital vein of both forearms. One cannula was used for frequent blood sampling, whilst the other was used to administer a stable isotope amino acid infusion. After obtaining a baseline blood sample, a primed continuous infusion of L-[ring- $^{13}\text{C}_6$] phenylalanine was initiated (prime: $2\ \mu\text{mol}\cdot\text{kg}^{-1}$; infusion: $0.05\ \mu\text{mol}\cdot\text{kg}^{-1}$, Cambridge Isotope Laboratories, Andover, MA, USA). The contralateral arm was warmed to $\sim 60^\circ\text{C}$ using an electric heating blanket to obtain arterialized blood samples at -180, -120, -60 and immediately prior to exercise, and at 20, 40, 60, 90, 120, 180, 240 and 300 min of post-exercise recovery. A total of 10 mL of arterialized blood was sampled at each time point and separated into ethylenediaminetetraacetic (EDTA) and serum separator vacutainers (BD, Oxford, UK). Blood samples were centrifuged for 10 min at 3000G and 4°C . Plasma and serum were aliquoted and stored at -80°C until further analyses. After ~150 min of stable isotope infusion, a muscle biopsy was obtained under local anaesthesia (1% lidocaine) from the quadriceps *vastus lateralis* of the non-dominant, non-exercised leg using the Bergström technique [30]. Muscle biopsy tissue was quickly rinsed in ice-cold saline and freed from any visible blood, connective tissue and fat before being snap-frozen in liquid nitrogen and stored at -80°C . Immediately after biopsy obtainment, participants completed a unilateral leg extension RE bout of the dominant leg to elicit a rise, but not maximise, MyoPS [31]. Resistance exercise consisted of two warm-up sets of 12 repetitions at 50% of their

previously estimated 1RM followed by 6 sets of 12 repetitions at 75% of their estimated 1RM, to elicit a rating of ~8-9 on the Borg CR-10 scale. Exercise sets were interspersed by 2 min of passive rest during which participants remained seated on the machine. Participants completed the exercise with a lifting-lowering cadence of ~1 sec without pause. Time-under-tension and Borg CR-10 rating were recorded after each exercise set. Following RE completion, participants consumed their respective PA or placebo control treatment over 1 h post-RE (described in detail below) and remained in a supine position for the remainder of the experimental trial. At 150 and 300 mins after starting treatment consumption, muscle biopsies were obtained from the non-exercised and exercised legs. Muscle biopsies were sampled ~2-3 cm from one another in a distal-to-proximal orientation. An overview of the experimental trial is provided in Figure 1.

Treatment Administration

Immediately and 60 min after RE completion, participants consumed two gelatine capsules (i.e. 4 capsules in total), each containing 375 mg of either a rice-flour placebo control (PL) or phosphatidic acid-enriched soybean phospholipid supplement (PA; Mediator® 50P, ChemiNutra, Austin, US). Treatment capsules were visually identical and ingested with water. The phospholipid composition of PA was; 50-60% phosphatidic acid, 5-15% phosphatidylcholine, 5-15% phosphatidylethanolamine, 1-5% phosphatidylinositol, 1-5% lyso-phosphatidylcholine, 1-5% N-acyl phosphatidyl ethanolamine. Thus, the total 1500mg of ingested supplement material provided ~750mg of PA over 1 h post-exercise. Supplements were ingested post-RE completion to minimize any potential negative effects of RE on gut absorption kinetics and minimize splanchnic extraction of PA [32] (i.e. absorption by intestinal mucosa). Based on the data of Purpura et al. [33] and our own pilot work in younger males, PA was ingested in a biphasic manner to ensure plasma PA concentrations

remained elevated for the majority of the RE recovery period. The PA supplement source and total ingested dose was similar to that used by others demonstrating; i) robust increases in p70S6K signaling in cultured cells [25], ii) elevated rates of MPS and mTORC1-mediated signaling in rats when consumed acutely in a similar equivalent dose for humans [23], iii) an increase in circulating PA and lysophosphatidic acid concentrations in a young male [33] and iv) augmented lean body mass and strength increases when consumed during prolonged resistance training in young males [25, 26], albeit using a different ingestion pattern to that chosen here.

Blood Analyses

Plasma amino acids, isotope enrichment and serum insulin

Plasma [$^{13}\text{C}_6$] phenylalanine enrichment was determined by gas chromatography-mass spectrometry (GCMS; model 5973; Hewlett Packard, Palo Alto, CA, USA) by monitoring ions 234/240. Briefly, 300 μL of plasma was diluted 1:1 with acetic acid before being purified through cation-exchange columns and dried down overnight under nitrogen. The purified amino acids were then converted to their N-tert-butyldimethyl-silyl-N-methyltrifluoroacetamide (MTBSTFA) derivative. Simultaneously, leucine and phenylalanine concentrations were measured by GCMS using the internal standard method based on the known volume of plasma and internal standard added and the known amino acid concentration of the internal standard. The internal standards were U- $^{13}\text{C}_6$ leucine (ions 302/308) and U- $^{13}\text{C}_9$ - ^{15}N phenylalanine (ions 336/346) added in a ratio of 100 $\mu\text{L}\cdot\text{mL}^{-1}$ of blood. Insulin concentrations were measured using commercially available enzyme-linked immunosorbent assay kit (IBL International, Hamburg, Germany).

Plasma phosphatidic acid concentration

Plasma [PA] was determined in a subset of participants (PA: n = 4; PL: n = 4) using the internal standard method, where 0.0013 mg of C17:0 PA (Avanti Polar Lipids, #830856) was added to 500 mL of plasma. Plasma lipids were extracted according to Folch et al [34] using a chloroform, methanol and acidified salt solution (2:1:0.8 v/v). Once extracted, lipids were re-dissolved in chloroform:methanol (2:1 v/v) before being spotted onto an HPTLC silica plate and separated into its component lipid fractions using a chloroform, petroleum ether, methanol, acetic acid and boric acid solvent mix (40:30:20:10:1:8 v/v). The PA position was identified using a TLC identification standard (Larodan AB, #37-0140 Phosphatidic Acid (egg PC)) before being removed and esterified, worked up and separated into fatty acids by gas chromatography (Agilent 7890 Gas Chromatograph with FID Detector, Agilent UK) using a carbowax column (Agilent, CP-Wax52CB). Plasma [PA] concentrations are expressed as $\mu\text{g}/\text{mg}$ plasma and fold-change from basal values, due to inherent background variability in [PA] between participants.

Muscle Tissue Analyses

Myofibrillar protein enrichment

The myofibrillar protein fraction and muscle free pool were extracted for the analysis of $^{13}\text{C}_6$ phenylalanine enrichment from ~30 mg of muscle tissue as previously described [35]. Briefly, muscle tissue samples were homogenised in a 2 mL microtube using clean sharp scissors. Homogenised samples were placed on a shaker (IKA, Vibrax, Germany) for 10 min at room temperature at 1500 rpm and subsequently centrifuged at 11000 g for 15 min at 4°C. The supernatant containing the amino acid free pool (IC) was transferred to a clean microtube and the myofibrillar pellet washed twice through centrifugation at 11000 g for 15 min at 4°C with 500 μL homogenisation buffer. To separate the collagen fraction from the myofibrillar pellet, the pellet was incubated in 750 μL of 0.3 M NaOH for 30 min at 30°C, giving the

sample a vortex mix at 15 and 30 min. The sample was then spun at 13000 rpm for 10 min at 4°C and the supernatant transferred to a 4 mL glass collection tube. A 750 µL volume of 0.3 M NaOH was then added to the pellet, centrifuged at 13000 rpm for 10 min at 4°C and the supernatants combined. Myofibrillar proteins were then precipitated by adding 1 mL of 1 M PCA, which was centrifuged at 3200 rpm for 20 min at 4°C. The pellet was washed twice with 2 mL of 70% ethanol. The remaining myofibrillar pellet was hydrolysed at 110°C overnight in 1 mL of 0.05 M HCl and 1 mL of activated Dowex 50W-X8 100-200 resin (Bio-Rad laboratories INC, USA). Constituent amino acids of the myofibrillar fraction were purified on cation-exchange columns by eluting with 2 M NH₄OH and evaporating to dryness. Amino acids were then derivatised as their n-acetyl-n-propylester, and phenylalanine labelling determined by gas chromatography-combustion-isotope mass spectrometry (Delta Plus XP, Thermofisher Scientific, Hemel Hempstead, UK).

Intramuscular signaling

Western blot analyses were performed on the sarcoplasmic protein fraction obtained during myofibrillar protein isolation (described above). Ubiquitin protein conjugates were analysed using separate sarcoplasmic protein fractions with the addition of 2-chloroacetamide (Sigma-Aldrich, St. Louis, MO) to a final concentration of 100 mM. Sarcoplasmic protein content was determined by a DC protein assay before western blot aliquots of 2 µg protein per 1 µL were prepared in 4x Laemmli sample buffer and sucrose lysis buffer and subsequently boiled for 5 min. Equal amounts of protein (30 µg) were loaded onto 7.5–15 % gels and separated by SDS-PAGE for 1 h. Following electrophoresis, proteins were transferred onto a biotrace nitrocellulose membrane (Pall Laboratory, Portsmouth, U.K.) for 1 h at 100 V, or in regard to the ubiquitinated proteins, S6 ribosomal protein (rps6), (LC3 a/b) and Caspase-3 onto a polyvinylidene fluoride (PVDF) membrane for 2.5h at 60 V. Membranes were subsequently

blocked in 5 % milk for 1 h and washed 3 times for 5 min in TBST before being incubated overnight at 4°C in following primary antibodies: Muscle Ring Finger protein 1 (MuRF1: Santa Cruz Biotechnology, Dallas, Texas, U.S; sc-398608), mono- and polyubiquitinated conjugates (FK2: Enzo Life Sciences LTD, Exeter, U.K.) phospho-p70S6K1 Thr389 (#9205), total 70 kDa S6 protein kinase (p70S6K1; #9202), phospho-eukaryotic initiation factor 4E binding protein (4E-BP1) Thr37/46 (#9459), total 4E-BP1 (#9452), phospho-eukaryotic elongation factor 2 (eEF2) Thr56 (#2331), total eEF2 (#2332), phospho-protein kinase B (Akt) Ser473 (#3787), total Akt (#9272), phospho-AMP activated protein kinase α (AMPK α) Thr172 (#2535), total AMPK α (#5831), phospho-p44/42 MAPK (Erk 1/2)^{Thr202/Tyr204} (#4370), total p44/42 MAPK (#4695), phospho-rps6 Ser240/244, total rps6 (#2217), LC3a/b (#12741) and Caspase-3 (#9665) each purchased from Cell Signaling Technology (New England Biolabs (UK) Ltd, Hitchin, U.K.). Membranes were then washed 3 times for 5 min in TBST and incubated for 1 h in their respective secondary antibody and washed again 3 times for 5 min in TBST. Protein quantification was achieved by incubating the membranes for 5 min in Immobilon Western chemiluminescent HRP substrate (Merck Millipore, Watford, UK) before being imaged using a G:BOX Chemi XT4 imager using GeneSys capture software (Syngene, Cambridge, U.K.). Bands were quantified using Image Studio Lite (Li-Cor, Lincoln, Nebraska, U.S.).

Calculations

MyoPS rates were calculated from $^{13}\text{C}_6$ phenylalanine incorporation by using the standard precursor-product model:

$$FSR (\% \cdot h^{-1}) = \Delta E_b / E_p \times 1 / t \times 100$$

Where ΔE_b is the difference in bound $^{13}\text{C}_6$ phenylalanine enrichment between two biopsy samples, E_p is the mean plasma precursor enrichment and t is the time in min between muscle

biopsy samples. Basal MyoPS rates were calculated in the rested/fasted state using the pre-infusion plasma $^{13}\text{C}_6$ phenylalanine enrichment as a proxy for basal muscle protein enrichment, a technique that has previously been validated in tracer naïve older individuals [36].

Statistics

Data analysis was performed using SPSS version 22 (IBM, Chicago, IL, USA). Anthropometric and exercise parameters were analysed using a one-way ANOVA. MyoPS rates, intracellular signaling, plasma PA concentrations and insulin were analysed using a two-way, repeated measures ANOVA with one within (three levels; basal, early and late MyoPS) and one between factor (two levels; group). A Tukey's HSD post hoc test was performed whenever a significant F ratio was found to determine specific differences. Significance was set at $p < 0.05$ for all analyses. All values are presented as means \pm SD or SEM.

RESULTS

Anthropometric and Exercise Variables

There were no significant differences between PA and PL for anthropometric or strength characteristics (Table 1). Similarly, RE variables including knee extension 1RM ($P = 0.43$), total time-under-tension ($P = 0.61$), time-under-tension per set ($P = 0.43$), total volume ($P = 0.46$), volume per set ($P = 0.46$) and average load per set ($P = 0.43$) were not significantly different between PLA and PA (Table 1).

Plasma Amino Acid, Phosphatidic Acid and Serum Insulin Concentrations

Plasma leucine and phenylalanine concentrations did not change from basal values during the experimental trial and were similar between groups at all time-points (Figure 2A and B, respectively). Serum insulin concentration decreased significantly below basal values by 90 min post-exercise cessation until 240 and 300 min post-exercise in PL ($P < 0.05$) and PA ($P < 0.05$), respectively, with no differences between groups (Figure 2C). Plasma $^{13}\text{C}_6$ phenylalanine enrichment significantly increased above basal values (-180 min) 60 min after initiation of the stable isotope tracer infusion and remained elevated for the duration of the trial in PL and PA ($P < 0.001$). Linear regression analysis revealed that the $^{13}\text{C}_6$ phenylalanine enrichment slopes in both groups were not significantly different from zero, confirming the obtainment of an isotopic steady state (Figure 2D). Plasma PA concentration was significantly elevated above basal values at 180 min post-exercise ($P = 0.02$) until 300 min post-exercise ($P = 0.01$) in PA only. There was no difference from basal plasma [PA] values in PL (Figure 3A and B). Fold-change in plasma [PA] concentration was significantly greater in PA compared with PL at 240 and 300 min post-exercise ($P = 0.026$ and 0.020 , respectively).

Myofibrillar Protein Synthesis

Basal-state, temporal MyoPS rates (0-150 min and 150-300 min) and aggregate MyoPS rates (0-300 min) in rested and exercised legs are presented in Figure 4A and 4B. MyoPS rates in the rested leg did not differ from basal values at any time-point, nor between groups. In the exercised leg, MyoPS rates over 150-300 min were significantly greater than basal values ($P = 0.001$) and 0-150 min ($P = 0.019$) in PL only, and were ~40% greater than basal values over the aggregate 0-300 min in PL only ($P = 0.023$). In the exercised leg, there was a strong trend for greater MyoPS rates over 150-300 min in PL compared with PA ($P = 0.051$). Temporal and aggregate MyoPS rates did not differ from basal values in rested or exercised

legs in PA, although there was a tendency for a reduction in MyoPS from basal values in the rested leg over 150-300 min in PA only ($P = 0.053$).

Intramuscular Signaling

Anabolic Signaling

Phosphorylation of p70S6K^{Thr389}, 4EBP1^{Thr37/46}, Akt^{Ser473} and rps6^{Ser240/244} in the PL exercised leg were, respectively, elevated 3.6-, 1.7-, 2.4- and 2.2-fold compared with baseline values at 150 min ($P = 0.002$; $P = 0.019$, $P < 0.001$ and $P = 0.021$ respectively), and remained significantly elevated 300 min post RE ($P = 0.003$; $P = 0.001$, $P = 0.025$ and $P = 0.007$ respectively). In the PL rested leg, AMPK α ^{Thr172} phosphorylation at 300 min was, respectively, 1.7- and 1.9-fold greater than corresponding values at baseline ($P = 0.028$) and 150 min ($P = 0.002$). At 150 min, p70S6K^{Thr389}, Akt^{Ser473} and rps6^{Ser240/244} phosphorylation in the PL exercised leg were greater than corresponding resting values at 150 min ($P = 0.002$, $P = 0.002$ and $P = 0.004$ respectively). At 300 min, 4EBP1^{Thr37/46}, p70S6K^{Thr389} and Akt^{Ser473} phosphorylation in the PL exercised leg were elevated compared with the corresponding rested leg ($P = 0.002$, $P = 0.001$ and $P = 0.009$ respectively). In the PA group, p70S6K^{Thr389} phosphorylation was significantly elevated from basal values 150 min upon exercise completion ($P = 0.047$). Rps6^{Ser240/244} was 2.1-fold elevated in the PA exercised compared with the rested leg at 150 min ($P = 0.037$), whilst AMPK α ^{Thr172} was 1.9-fold greater in the exercised compared with rested leg at 300 min ($P = 0.027$). Between-group differences were observed at 150 min in the rested leg for 4E-BP1^{Thr37/46} ($P = 0.022$) and in the exercised leg for Akt^{Ser473} ($P < 0.001$), whilst between-group differences at 300 min were apparent in the rested leg for AMPK α ^{Thr172} ($P = 0.001$) and in the exercised leg for p70S6K^{Thr389} ($P = 0.046$), 4EBP1^{Thr37/46} ($P = 0.005$) and rps6^{Ser240/244} ($P = 0.003$) phosphorylation. No changes were found for MAPK^{Ser44/42} and eEF2^{Thr56} phosphorylation (Figure 5A-F).

Proteolytic Signaling

MuRF1 protein content (Figure 6A) was elevated 1.2-fold from baseline values in the PA rested leg at 300 min ($P = 0.019$), and 1.4-fold in PL exercised leg at 300 min compared with their corresponding resting value ($P = 0.002$). Between-group differences for Murf1 protein content occurred in the rested leg at 300 min ($P = 0.019$). Total ubiquitinated protein conjugates (Figure 6B) in the PL rested leg were, respectively, elevated 1.4- and 1.3-fold above basal values at 150 ($P = 0.001$) and 300 min ($P = 0.005$), and were greater than corresponding exercising values at 150 ($P = 0.008$) and 300 min ($P = 0.007$). Between-group differences were apparent for the resting leg at 150 ($P < 0.001$) and 300 min ($P = 0.002$). Quantification of a single distinct band at ~30 kDa (Figure 6C) revealed significantly elevated total protein ubiquitination in the PL exercised leg at 150 min compared with basal values ($P = 0.011$) and corresponding resting values ($P = 0.047$; Figure 6B). Ubiquitinated proteins seemed to occur mainly above 100 kDa. LC3 II protein content (Figure 6D) was elevated from baseline values 150 min post exercise in PL ($P = 0.027$). Higher LC3 II protein contents were found in PL compared with PA at 150 ($P < 0.001$) and 300 min ($P = 0.015$) in the exercising leg, and at 150 min in the resting leg ($P = 0.003$). Total Caspase-3 (Figure 6E) protein decreased in the PL resting leg from 150 min to 300 min ($P = 0.022$).

DISCUSSION

At the forefront of strategies to combat sarcopenia is the development of novel interventions to enhance MyoPS and overcome the ‘anabolic resistance’ of aged skeletal muscle. In this regard, PA has been touted as a nutraceutical with muscle anabolic properties, phosphorylating mTORC1-mediated signaling leading to enhanced protein translational efficiency [24, 27]. Both endogenous PA production [37] and exogenous PA provision [22]

phosphorylate mTORC1 and associated distal signaling proteins (i.e. p70S6K and 4E-BP1) in cell culture and animal models, albeit via different pathways. Herein, we aimed to investigate these separate but synergistic effects of distinct PA-mediated signaling pathways on MyoPS in older individuals through acute oral PA provision alone or combined with mechanical loading. Following PA ingestion, plasma [PA] concentrations rose above basal values at 180 min post-exercise. Surprisingly, PA ingestion inhibited the RE-induced rise in intramuscular anabolic signaling and MyoPS observed in PL over 5 h of recovery. No robust group interactions were found, likely due to the relatively small sample size studied. Thus, our findings suggest that, in an acute experimental setting, oral PA provision has an interference effect on the muscle remodelling response to RE in older individuals.

Reconciling the present findings of a potential PA-induced interference effect on MyoPS is difficult considering the established *in vitro* evidence that PA exerts anabolic effects on mTORC1-mediated signaling [22, 25, 38]. Support for our findings can be gleaned from the work in rodents [23], in which a whey protein-induced rise in MPS was attenuated by co-ingestion of PA, at a dose equivalent to that provided herein. Despite dampened MPS rates with PA co-ingestion, mTORC1-mediated signaling events were numerically greater with combined whey protein *plus* PA. The authors suggested PA may have altered mTORC1-mediated signaling, causing the peak MPS time to shift. The results of our study support similar inhibitory effects of PA on MyoPS, particularly in the presence of a second anabolic stimulus (i.e. RE). In contrast to the work of Mobley et al. [23], our findings demonstrate that acute oral PA ingestion prevented any RE-induced rise in anabolic signaling, which most likely impaired protein translational efficiency and prevented a similar MyoPS response to that observed in PL [39]. Elucidating the mechanisms underlying the tendency towards lower resting MyoPS rates over 150-300 min post-PA ingestion is more challenging. These observations are in stark contrast to Mobley et al. [23], in which PA ingestion alone increased

anabolic signaling and potentially MPS. These discrepant findings could be attributed to the different species studied (rodents vs. humans). Besides PA-related alterations in downstream mTORC1-mediated signaling, acute oral PA supplementation did not alter the ERK signaling pathway, which has been suggested to be an important mechanistic link between exogenous PA provision and mTORC1-mediated signaling *in vivo* [40]. The PA-induced activation of ERK, and thus mTORC1 signaling, hinges on the conversion of PA to lysophosphatidic acid (LPA), which, in turn, depends on the presence of the converting enzyme phospholipase A (PLA) [22]. Thus, the absence of between group differences in MAPK signaling, in response to exogenous oral PA ingestion, suggests that this route of administration may not have altered LPA production. Taken together, the present findings demonstrate an impaired anabolic signaling response to RE when combined with acute PA ingestion, reinforcing the lack of any MyoPS response.

In addition to MyoPS and anabolic signaling, changes in proteolysis and proteolytic signaling events may play an important role in the overall net muscle protein balance to RE [41]. Therefore, we were interested to see if acute oral PA ingestion modulated any potential change in RE-induced proteolytic signaling. Earlier work conducted by Jaafar et al. [42] revealed distinctive anti-proteolytic characteristics for PA. Specifically, overexpressing myotubes with the PA precursor, phospholipase D1, protected against the atrophy-promoting agent dexamethasone, whilst PA provision protected against the atrophying effects of TNF- α . In our *in vivo* human ageing model, we found equivocal effects of oral PA ingestion on intracellular proteolytic signaling. Whereas Caspase-3 did not reveal any significant between group differences, 30kDa Ubiquitin-conjugates and LC3-II seemed to be higher in PL compared with PA. However, it is important to acknowledge that others have previously observed that alterations in the ubiquitin proteasome pathway are not easily reconciled with observed effects on muscle protein turnover [43]. Furthermore, the proteolytic markers

measured in the present study only represent a small fraction of the vast complexity of the signaling proteins that regulate proteolysis and offer only snapshots of this dynamic processes. Without direct measurement of myofibrillar breakdown rates, which is technically challenging, the effects of orally ingested PA on proteolysis remain inconclusive

The specific mechanisms through which PA interferes with RE-induced MyoPS and potentially impairs basal-state MyoPS in older individuals are intriguing. One possibility is that PA-derived production of diacylglycerol, catalysed by the enzyme phosphatidic acid phosphatase, may impair insulin signaling and blunt MyoPS rates [44-46]. Indirect evidence for lipid-induced impairment in MyoPS is apparent from our recent findings of a negative association between fat mass and postprandial MyoPS rates in older individuals [47]. Furthermore, Stephens et al. [48] reported a suppression in postprandial MPS rates and mTORC1-mediated signaling (i.e. anabolic resistance) when insulin resistance was induced via lipid infusion in healthy young individuals. It is important to note that Stephens et al. [48] infused lipid intravenously over 7 h, bypassing splanchnic absorption, resulting in a circulating lipid milieu that was substantially greater and occurred more rapidly than our acute oral PA ingestion protocol. Furthermore, intravenous lipid infusion resulted in significant intramuscular lipid metabolite accumulation, which would directly explain impairments in insulin signaling and MPS. In the present study, acute oral PA ingestion significantly increased plasma [PA] ~2-3-fold from 180 min post-ingestion, which coincided with the temporal impairment in MyoPS over 150-300 min post-exercise. Unfortunately, due to limitations on biopsy tissue sampling, we can only speculate as to whether the increase in plasma [PA] following acute oral PA ingestion altered intracellular lipid content and insulin resistance. What is clear, is that events arising from the extracellular increase in [PA] were sufficient to impair mTORC1-mediated signaling and MyoPS.

Although we have provided the first *in vivo* mechanistic investigation of orally ingested PA in humans, there is still much to be understood about the muscle anabolic properties of this purported nutraceutical. Centred around previous animal studies establishing the muscle anabolic properties of acute PA consumption [23], we chose to administer a similar equivalent oral dose based on the per species conversion calculations of Reagan-Shaw et al [49], in an attempt to understand whether these responses would translate to an *in vivo* human ageing model. The delayed rise in plasma [PA] following PA ingestion, compared with case study values reported by others [33], might be due to the biphasic ingestion protocol, where the second dose of PA was ingested 60 min after the first. However, we acknowledge that the timing of PA ingestion around RE may influence any potential anabolic effects of this compound. Indeed, enhancements in RE-induced strength and lean body mass in young males were reported when supplemental PA was consumed prior-to (450mg) and immediately after (300mg) RE training [25], although others failed to replicate these findings [50]. In theory, replicating the PA ingestion pattern of Joy et al. [25] would have shifted the increase in plasma [PA] above basal values to ~60 min earlier than observed (i.e. 120 min post-exercise), which, we posit, is unlikely to have significantly altered MyoPS responses. Nonetheless, given that plasma [PA] concentrations remained elevated above basal levels at 5 h post-RE, we cannot rule out MyoPS responses >5 h post-RE might have differed between PL and PA. In light of evidence of the importance of contraction-induced elevations in intracellular [PA] for mTORC1 activation, and the reported anabolic properties of prolonged PA supplementation [25, 51], our finding of an inhibitory effect of acute PA ingestion could indicate that longer-term supplementation may be necessary to increase in intracellular [PA] and muscle anabolism in older individuals. In support of this notion, supplementation of lipid-based nutritional compounds, omega-3 polyunsaturated fatty acids and arachidonic acid, over 4-8 weeks has been reported to alter

intracellular lipid composition and modulate the acute anabolic signaling and ribosome biogenesis response to RE in young individuals [52, 53]. Thus, an important next step is to investigate whether longer-term PA supplementation alters intramuscular [PA] and enhances the acute MyoPS response to RE in older individuals, in addition to better understanding of how supplemental PA dosing and ingestion pattern can be altered to ‘optimize’ delivery.

In summary, this is the first *in vivo* study to characterise the effects of acute oral PA ingestion on MyoPS and intramuscular signaling at rest and post-RE in older individuals. Oral PA ingestion attenuated the RE-induced increase in MyoPS. The precise cause of the apparent interference effect of oral PA ingestion on RE-induced muscle anabolism in older individuals remains to be fully elucidated, but seems to be underpinned by impaired intramuscular anabolic signaling. Based on our acute mechanistic findings, oral PA ingestion does not appear to be an effective means of enhancing the muscle anabolic response to RE in older individuals. However, longer-term supplementation studies are required to fully understand whether this purported nutraceutical holds any anabolic potential for aged skeletal muscle.

Disclosures

The authors have no conflicts of interest to declare.

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Authorship Statement

All authors gave their final approval of the version of the article to be published. BS, PJA and LB designed the study. BS and LB organized and carried out the clinical experiments with the assistance of JM. BS, YN, JM, ML, KS, PJA and LB performed all data analyses. BS, and LB performed the statistical analysis of the data. BS, PJA and LB wrote the manuscript together. BS and LB are the guarantors of this work and take responsibility for the integrity and accuracy of the data analysis.

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TABLES

Table 1. Participant anthropometric, strength and resistance exercise characteristics.

	PL (n = 8)	PA (n = 8)
Age (yrs)	69.4 ± 3.6	68.4 ± 1.8
Age range (yrs)	65 – 75	66 – 70
Body mass (kg)	73.9 ± 7.4	73.7 ± 8.9
BMI (kg·m ⁻²)	23.1 ± 1.6	24.4 ± 2.1
Whole-body FM (kg)	16.4 ± 2.9	19.4 ± 4.0
Leg FM (kg)	5.2 ± 1.0	5.9 ± 1.5
Whole-body FFM (kg)	53.6 ± 5.7	50.6 ± 5.6
Leg FFM (kg)	17.1 ± 1.4	16.1 ± 1.9
Knee extension 1RM (kg)	87.4 ± 18.3	79.4 ± 20.7
Training volume (kg)	1918 ± 377	1725 ± 602
Volume per set (kg)	320 ± 63	288 ± 100
Time-under-tension total (sec)	183 ± 37	170 ± 66
Time-under-tension per set (sec)	31 ± 6	28 ± 11
Average load per set (kg)	26.9 ± 4.3	24.3 ± 8.2
Average Borg CR-10	8.4 ± 1.4	8.1 ± 1.5

Values are presented as means ± SD. BMI; body mass index, FM; fat-mass, FFM; fat-free mass, 1RM; one-repetition maximum, Borg CR-10; Borg category-ratio scale.

FIGURES

Figure 1: Schematic overview of the experimental trial. PA; 750mg of phosphatidic acid-enriched soybean phospholipid supplement, PL; 750mg of rice-flour placebo.

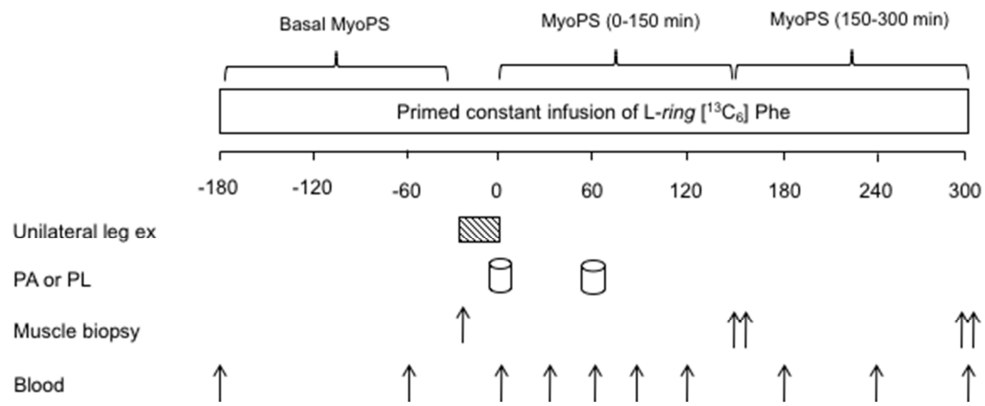
Figure 2: Plasma phenylalanine (A), leucine (B) and insulin concentration (C) and $^{13}\text{C}_6$ phenylalanine enrichment (D) in experimental trials. At $t = 0$ and 60 min, participants orally ingested 750mg of PA or PL with water. * indicates significantly different from basal fasting values (-180 min) for PL and PA. † indicates significantly different from basal fasting values for PA only. Values are means \pm SEM. Significance was set at $P < 0.05$.

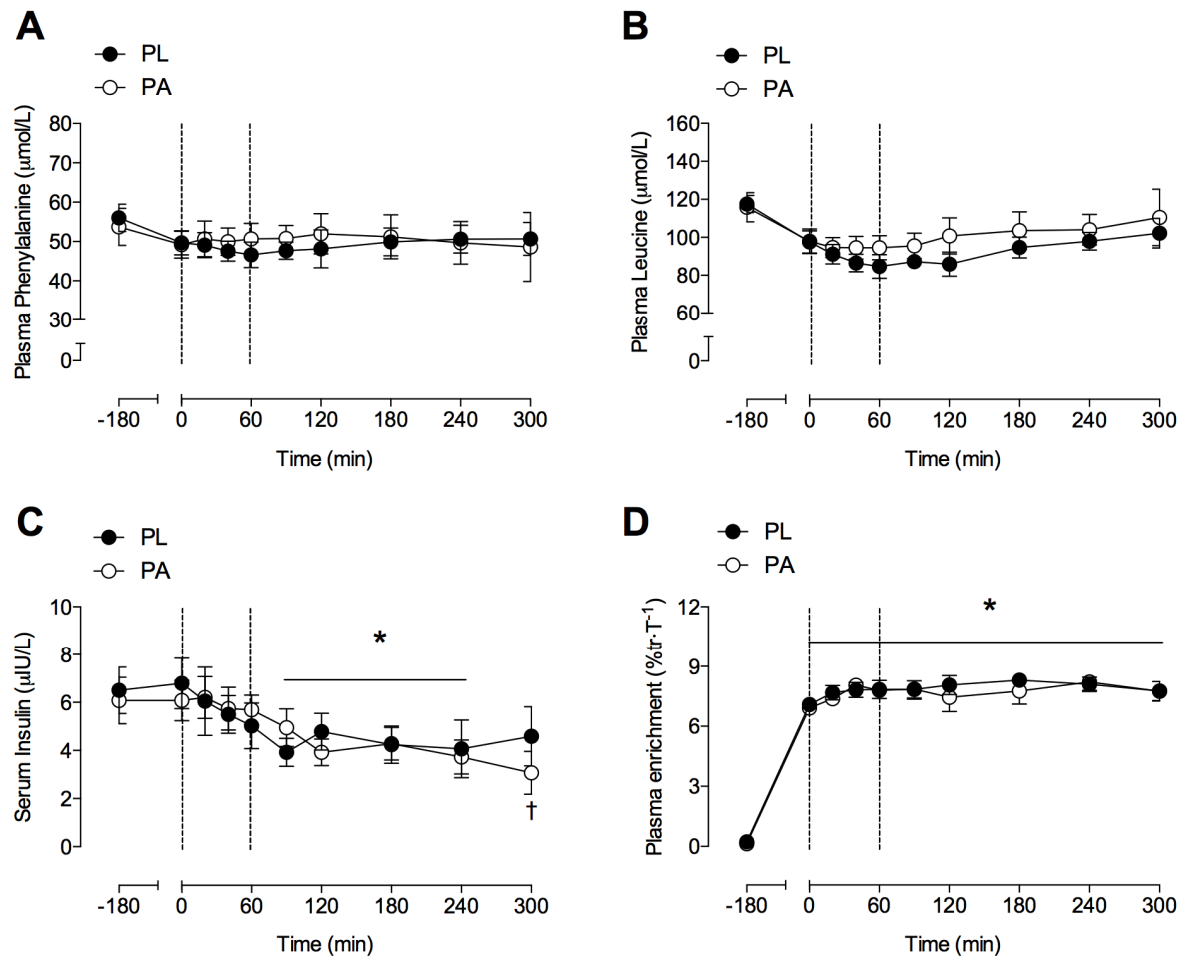
Figure 3: Plasma PA concentrations expressed as absolute values (A) and fold-change from basal (B). At $t = 0$ and 60 min, participants orally ingested 750mg of PA or PL with water. Subscript *a* indicates significantly greater than basal values (0 min) for PA. Subscript *b* indicates significantly greater than values at 60 min for PA. * indicates significant between-group difference at the same time-point. Values are means \pm SEM. Significance was set at $P < 0.05$.

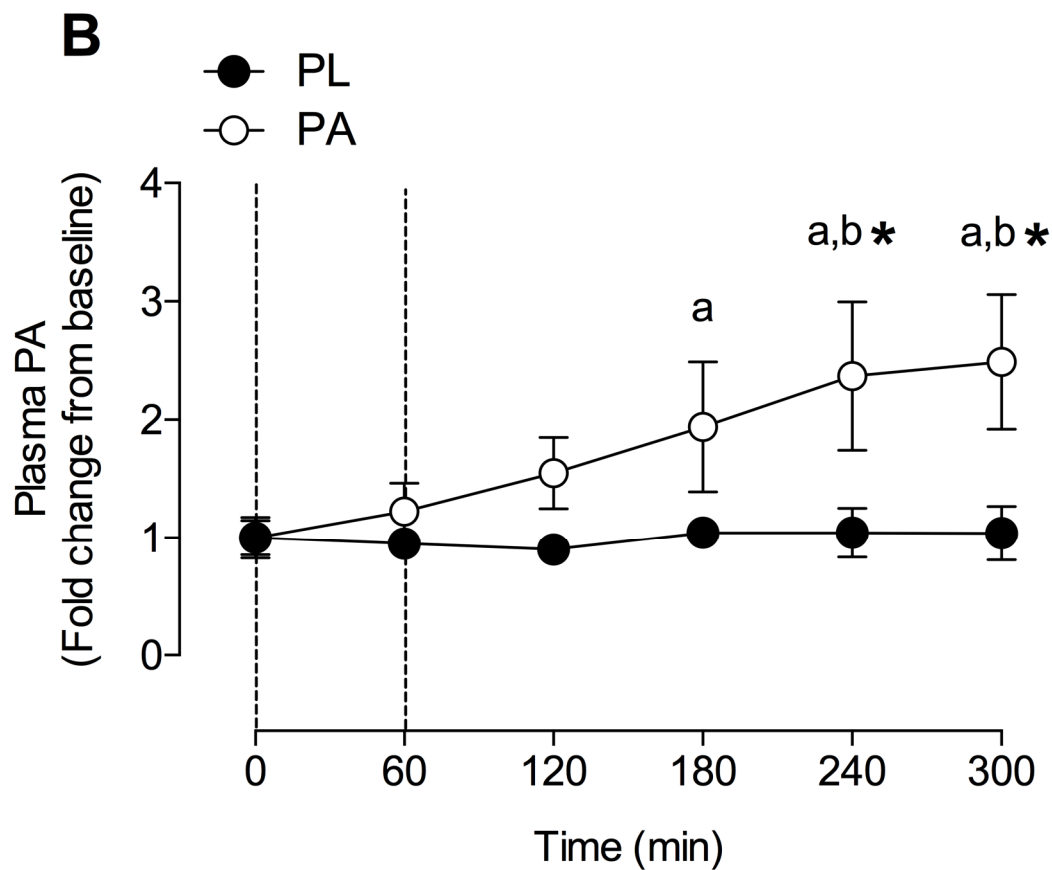
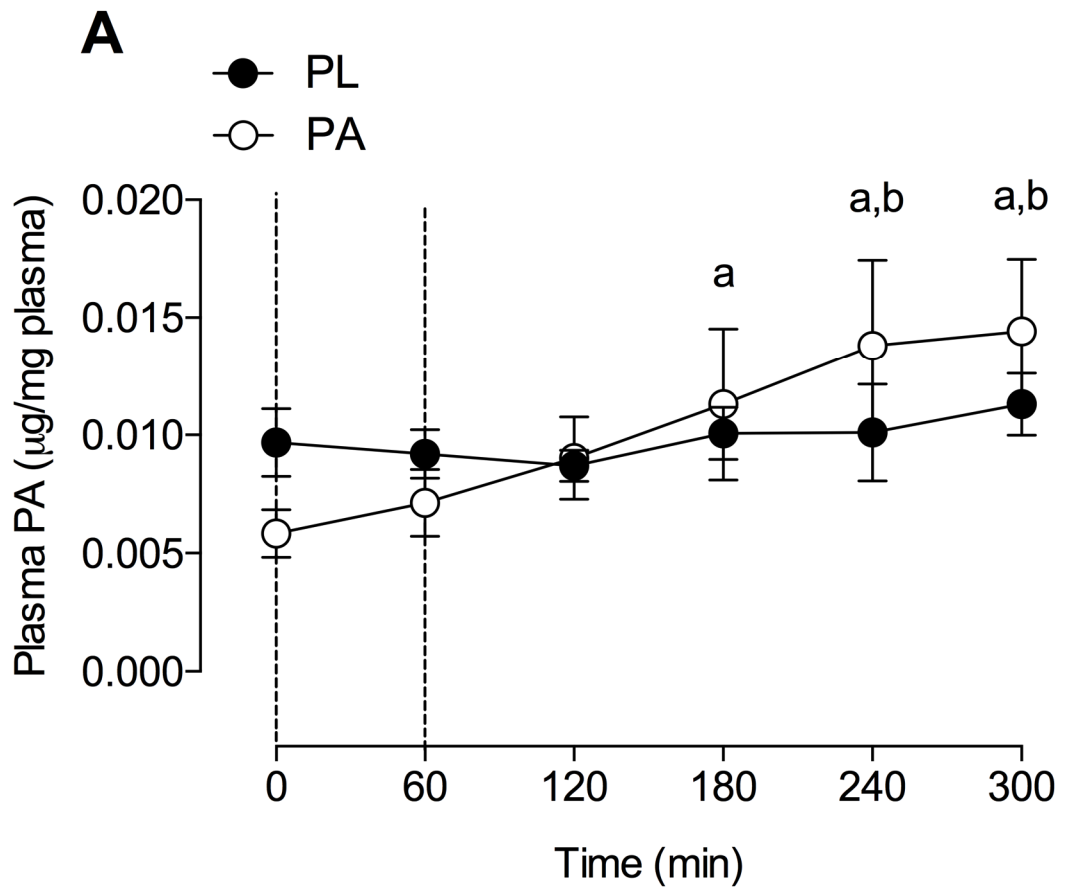
Figure 4: Myofibrillar fractional synthesis rate (FSR) during basal postabsorptive conditions (prior to exercise and/or treatment administration) and over the 0-150 and 150-300 or aggregate 0-300 min post-exercise recovery period in the resting leg (REST; A) and exercised leg (EX; B) after oral ingestion of PA or PL. * indicates significantly greater than basal state MyoPS rates in the same leg, † indicates significantly greater than MyoPS rates over 0-150 min in the same leg. Values are means \pm SEM. Significance was set at $P < 0.05$.

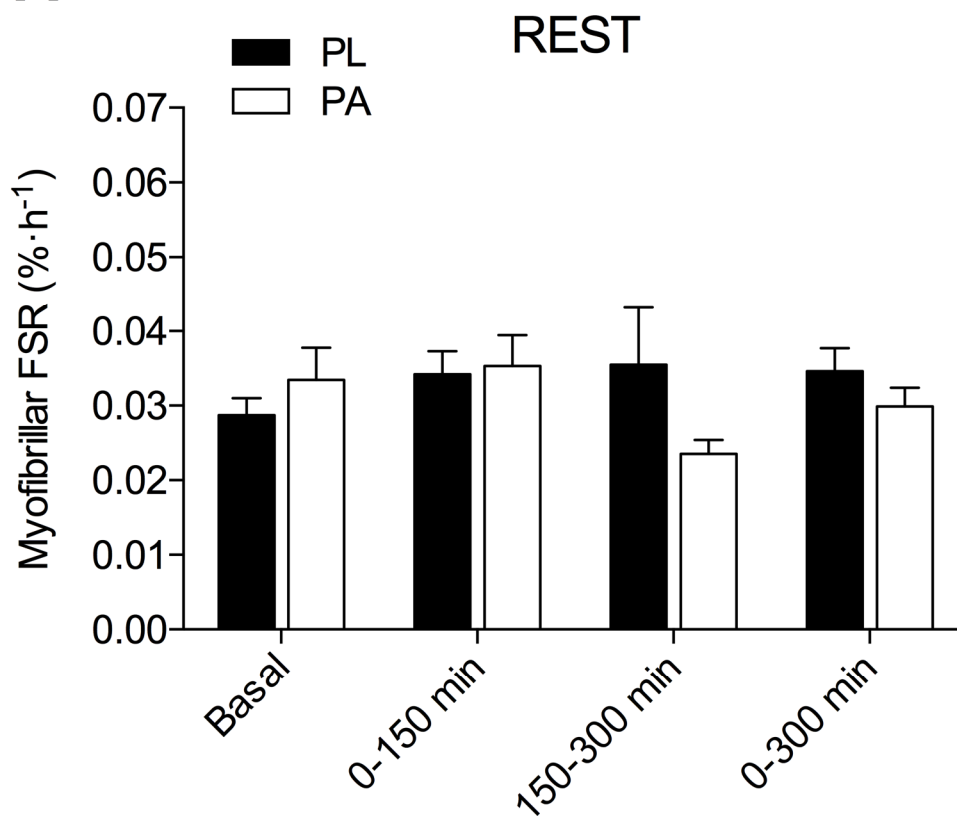
Figure 5: Intracellular signaling phosphorylation of p70S6K^{Thr389} (A), 4E-BP1^{Thr37/46} (B), Akt^{Ser473} (C), eEF2^{Thr56} (D), ERK1/2^{Thr202/Tyr204} (E) and AMPK^{Thr172} (F) expressed as fold-change from basal values at 150 and 300 min post-exercise in rested and exercised legs with PA and PL ingestion. * indicates significantly greater than basal value, ‡ indicates significant within-treatment difference from value at 150 min, † indicates significant different from rested leg at the same time-point, # indicates significant between-treatment difference at same time-point. Values are presented as means \pm SEM. Significance was set at $P < 0.05$.

Figure 6: Intracellular proteolytic markers for the abundance of MuRF1 (A), total ubiquitin conjugates (B) ubiquitinated proteins at 30 kDa (C), LC3 (D) and Caspase-3 (E) expressed as fold-change from basal, normalized to Ponceau. * indicates significantly greater than basal value, † indicates significant different from rested leg at the same time-point, # indicates significant between-treatment difference at same time-point. Values are presented as means \pm SEM. Significance was set at $P < 0.05$.







A**B**